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Induction of Phenylalanine Ammonia-Lyase and Increase in Phenolics in Lettuce Leaves in Relation to the Development of Russet Spotting Caused by Ethylene[1]

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HIROSHI HYODO,[2] HIROYUKI KURODA,[3] AND SHANG FA YANG
Department of Vegetable Crops, University of California, Davis, California 95616

ABSTRACT

Russet spotting (RS), consisting of numerous small brown spots on the midrib of head lettuce (*Lactuca sativa*), is a physiological disorder induced by exposure to ethylene. In leaves suffering RS, the increase in spotting was accompanied by a parallel increase in the amount of phenolic compounds. Of these, chlorogenic acid and isochlorogenic acid were identified. Ethylene induced high phenylalanine ammonia-lyase (PAL) activity and RS formation in the susceptible cultivar Salinas, but not in the resistant cultivar Calmar. In the absence of ethylene neither significant PAL induction nor RS occurred. No correlation was found between the increase in polyphenol oxidase or peroxidase and the development of RS. The increase in PAL activity, however, was closely correlated with the development of RS. The increase in PAL activity preceded the development of RS, and the extent of RS was directly related to the level of PAL. Three temperatures (0.5, 5.5, and 12.5 C) were compared on the basis of their influence on both RS and PAL induction. At the lowest temperature (0.5 C) neither PAL induction nor RS occurred to a significant extent. At the highest temperature (12.5 C) an initial rapid increase in PAL activity and an earlier development of spotting were observed, but subsequently there was a decrease in both PAL activity and the rate of development of RS. At the medium temperature (5.5 C) both PAL activity and RS increased progressively with time. The decline of PAL activity at a higher temperature might be attributed to inactivation of the enzyme. Thus, a temperature favorable for induction of PAL activity by ethylene was also favorable for RS. These observations indicate a close interrelationship between the induction of PAL activity and the development of RS in response to ethylene, and suggest a causal relationship between the two events. PAL serves as a useful biochemical marker for the RS reaction.

Russet spotting is one of the most common commercially important disorders of postharvest lettuce (*Lactuca sativa*). It is observed as numerous small brown spots along both sides of the midrib, and may spread over the leaf blade during senescence (16, 18). It is caused by exposure of the lettuce to ethylene (25). According to Morris *et al.* (19), the factors affecting RS[4] are: temperature (maximum susceptibility at near 5 C), ethylene (0.1 μl of ethylene caused RS within 3 days and the maximum effect was apparent after 7 days), O_2 (low O_2 inhibited RS), CO_2 (increasing concentrations inhibited RS), and maturity (firmer heads were more susceptible).

Lipton (17) and Ilker *et al.* (10) examined russet spots microscopically, and noted that discoloration spread to several subepidermal mesophyll cells as well as to the vascular tissue, and that the collapse of mesophyll cells resulted in pit-like depressions. Although RS has been the subject of detailed examination in respect to the symptomology and the various factors which influence the development (16–19, 25), no biochemical studies have been reported. Since the metabolism of phenolic compounds has often been thought to be involved in necrogensis, we have examined activities of five enzymes (PAL, CAH, OMT, PPO, and peroxidase) involved in phenol metabolism as possible biochemical markers, and have investigated the relationship between the activation of these enzymes and the development of RS caused by ethylene.

MATERIALS AND METHODS

"Crisphead"-type lettuce was used for all experiments. Cultivar Salinas was harvested at Salinas, California, and cv. Calmar was obtained at a local wholesale market. Harvested lettuce heads were stored at 0.5 C until used. Wrapper and cap leaves were removed and the next six to seven leaves were taken for experimental use. Ten to 12 leaves were placed in a 8.7-liter glass jar with a metal lid. A continuous flow of humidified air (air control) or air containing 1.2 μl/l of ethylene (ethylene treatment) at a flow rate of 10 liter/hr was applied to the leaves. Ethylene concentration was monitored by gas chromatography. Leaves were kept at 5.5 C except for those otherwise specified. The small russet-brown spots appeared mostly on the midrib of ethylene-treated leaves, but were also found on the blade. To describe the extent of injury, a rating scale of 1 to 9 was used. A score of 1 on this scale indicates no injury, and a score of 9 extreme injury.

After an appropriate period of incubation in either air or air plus ethylene, discs (9-mm diameter) were excised from the midribs of the leaves with a cork borer. For determination of phenolic content, the discs were blended in a Sorvall Omni-Mixer and extracted in 80% ethanol. The filtrate was assayed for phenolic content with a Folin-Ciocalteu phenol reagent (6) using *p*-coumaric acid as a standard.

For the identification of CHA and ICHA the ethanol was concentrated under reduced pressure and the concentrate was analyzed by paper chromatography and TLC. Fluorescence of CHA, ICHA, and other authentic polyphenolic compounds was observed under an UV lamp providing a maximal wavelength of 366 nm. For isolation of CHA and ICHA, ethylene-treated leaves showing severe symptoms of RS (score 7–9) were used. The

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[2] Present address: Department of Horticulture, Faculty of Agriculture, Shizuoka University, Okya, Shizuoka 422, Japan.

[3] Present address: Wood Research Institute, Kyoto University, Uji, Kyoto, Japan.

[4] Abbreviations: RS: russet spotting; PAL: phenylalanine ammonia-lyase; CAH: cinnamic acid-4-hydroxylase; OMT: O-methyltransferase; PPO: polyphenol oxidase; CHA: chlorogenic acid; ICHA: isochlorogenic acid.

concentrated extract was streaked on Whatman 3MM paper and developed with l-butanol-acetic acid-water (4:1:5, v/v). The bands corresponding to CHA and ICHA were cut out and eluted with 40 and 80% ethanol, respectively. After concentration these eluates were subjected to paper electrophoresis at pH 7 or 10 at 36 v/cm for 40 min. Further purification of CHA and ICHA fractions was carried out by paper chromatography developed with 5% acetic acid. TLC was carried out on cellulose plates with a solvent of ethyl acetate-acetic acid-water (9:2:2, v/v). CHA, ICHA, and caffeic acid were detected by fluorescence under UV light in the presence or absence of NH_3 vapor, and by the Hoepfner reaction (24). Phenolic spots were revealed by spraying with a mixture of $FeCl_3$ and $K_3Fe(CN)_6$. ICHA was a gift from J. Corse.

Enzyme Assays. Eight to 10 midrib discs (2–3 g) were homogenized with mortar and pestle in 10 ml of 0.1 M Tris-HCl buffer (pH 7.3) containing 5 mM 2-mercaptoethanol and 0.3 g of Polyclar AT. The homogenate was centrifuged at 12,000*g* for 20 min. Extraction and centrifugation were carried out in the cold (below 4 C). PAL activity of the supernatant was assayed by measuring the increase in absorption at 290 nm as described elsewhere (8); 1 unit is defined as the amount of PAL which produces 1 μmol of cinnamic acid in 1 hr under the specified conditions (8). The supernatant was also used for CAH and OMT assay by the procedure previously reported (7, 13). For the assays of PPO and peroxidase, the supernatant was dialyzed three times against 2 liters of 0.01 M K-phosphate for 8 hr to remove 2-mercaptoethanol. The PPO was assayed by following O_2 consumption in the presence of caffeic acid at 30 C with an O_2 electrode. The reaction mixture contained 7 μmol of caffeic acid, 0.5 mmol of K-phosphate (pH 6.5), and a suitable amount of enzyme (derived from less than 50 mg fresh wt of tissue) in a total volume of 3.1 ml. Peroxidase activity was measured by the rate of *o*-dianisidine oxidation, as described elsewhere (28).

All experiments were repeated more than once, and the data reported here represent the mean of duplicate samples in a typical experiment. Although there were some variations in PAL activities, the trends were identical.

RESULTS

With susceptible cv. Salinas, no symptoms of RS and little change in phenolic content were observed in the air control tissues (Fig. 1a). With ethylene treatment RS became apparent after a lag phase of 2 days, and after 7 to 8 days of incubation their RS scores reached 8 to 9 (Fig. 1b). From extracts of fresh leaf tissue (day 0), four spots were revealed by $FeCl_3$-$K_3Fe(CN)_6$. After incubation for 7 days in humidified air, two additional spots appeared, designated X and Y. These two spots were fluorescent. They were much larger and more intense in extracts from ethylene-treated tissue than in extracts from air controls. The R_F values and the fluorescence and Hoepfner color reactions of compounds X and Y were identical to those of authentic CHA and ICHA, respectively, when similarly chromatographed. Compounds X and Y were subsequently eluted from paper chromatograms and subjected to paper electrophoresis. The mobilities of X and Y were again equivalent to those of CHA and ICHA, respectively. The UV spectra of isolated X and Y were in strict accordance with those of CHA and ICHA. These results are summarized in Table I. After alkaline hydrolysis under nitrogen, both compounds gave rise to caffeic acid. It is believed that compounds X and Y are CHA and ICHA, respectively.

Preliminary experiments were conducted to examine the relationship between the development of RS and the activation of the enzymes (PAL, CAH, OMT, PPO, and peroxidase) involved in the phenylpropanoid pathway and in polyphenol oxidation in both susceptible cv. Salinas and resistant cv. Calmar. PAL was found to be closely related to the development of RS. CAH activity was found to be enhanced by ethylene treatment in both cultivars (data not shown), as has been found in other plant tissues

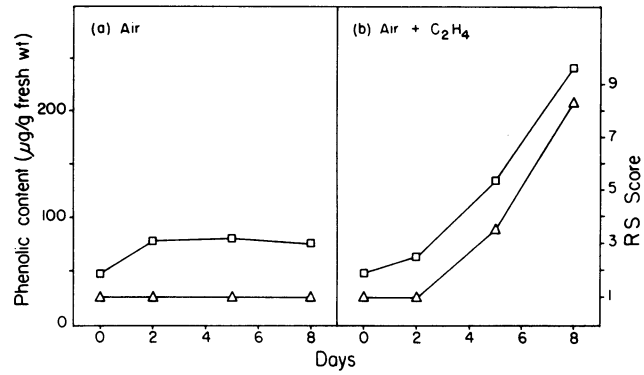

FIG. 1. Increase with time in the amount of phenolics and the development of russet spotting (RS) in leaves of cv. Salinas stored at 5.5 C in humidified air or in air plus ethylene (1.2 μl/l). Δ: RS score; □: phenolic content.

Table I. Comparison of compounds X and Y isolated from ethylene-treated lettuce leaves with chlorogenic and isochlorogenic acid.

	Comp X	CHA	Comp Y	ICHA
R_f				
PC[1]	0.66	0.66	0.80	0.80
TLC[2]	0.51	0.51	0.90	0.90
Fluorescence				
$-NH_3$	Blue white	Blue white	Blue white	Blue white
$+NH_3$	Green	Green	Yellow	Yellow
Hoepfner reaction				
-NaOH	Yellow	Yellow	Yellow	Yellow
+NaOH	Pink red	Pink red	Pink red	Pink red
PE mobility[3]	0.31	0.31	0.11	0.11
λ_{max} nm[4]	328	328.5	329	328.5

[1] Paper chromatography with 1-butanol:acetic acid:water (4:1:5, v/v).

[2] Thin layer chromatography on a cellulose plate with ethylacetate:acetic acid:water (9:2:2, v/v).

[3] Paper electrophoresis with 0.025 M K-phosphate buffer at pH 7. Mobility indicates distance migrated toward the anode relative to that of N-(2,4-dinitophenyl)cysteic acid.

[4] UV absorption was determined for each fraction separated by paper chromatography.

(7, 20). CAH activity, however, was not as closely correlated as PAL activity with the development of RS. Results for OMT (not shown) were similar to those for CAH. Although PPO increased during the incubation, no correlation was observed between the increase in PPO activity and the development of RS or ethylene treatment. Similar results were observed with peroxidase. These results indicate that neither PPO nor peroxidase could be rate-limiting in the regulation of RS development.

We have, therefore, focused our attention on the relationship between PAL induction and RS formation. Ethylene did not induce an increase in either RS or in PAL activity in the resistant cv. Calmar (Fig. 2). In the susceptible cv. Salinas, however, PAL activity increased markedly in ethylene-treated tissue as RS developed, and this increase in PAL appeared to precede the increase in RS (Fig. 3). In the absence of ethylene (air control), there was little change in either PAL activity or in the extent of RS. It is evident that the susceptibility of lettuce tissue to RS paralleled its capacity for the formation or activation of PAL in response to ethylene.

PAL activity was positively correlated with the number of RS lesions in 1-cm-diameter discs prepared from midribs of several heads of an unknown cultivar which had been treated with ethylene (Fig. 4). Those discs which contained few or no RS lesions were low in PAL activity, even though they were cut from an area adjacent to intense RS. Thus, the great variation in the number of RS lesions was paralleled by variation in PAL activity from one area to another.

It has been reported that the optimum temperature range for

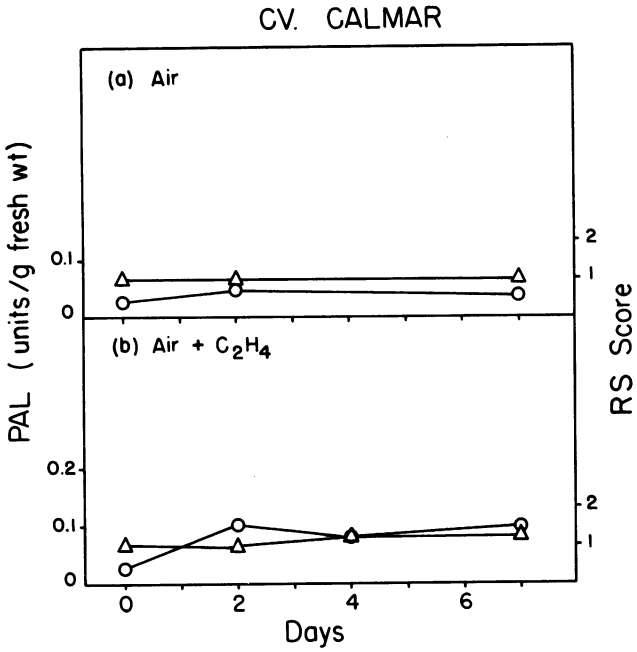

FIG. 2. PAL activity and RS score in leaves of cv. Calmar stored at 5.5 C in air (a), or in air plus ethylene (b). Δ: RS score; O: PAL activity.

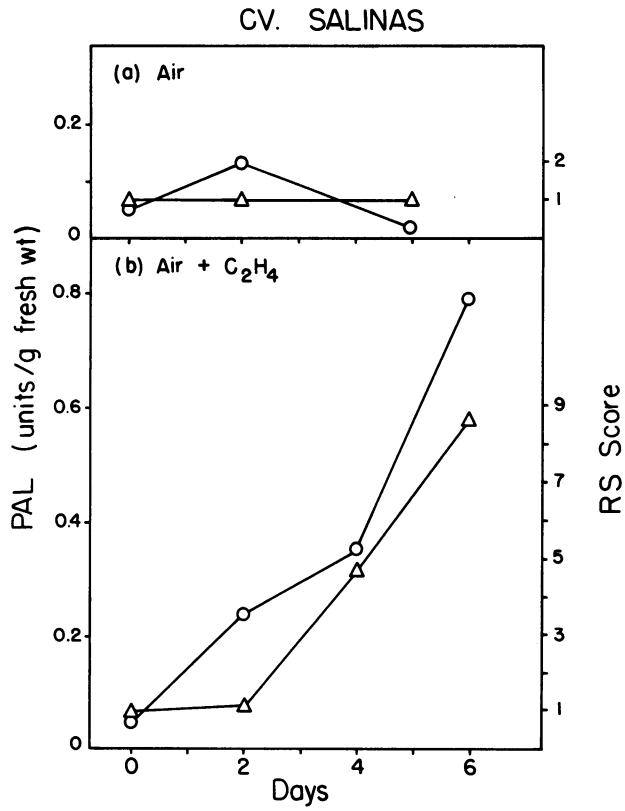

FIG. 3. Increase in PAL activity and development of RS in the leaves of cv. Salinas stored at 5.5 C in air (a), or in air plus ethylene (b). Δ: RS score; O: PAL activity.

RS is 5 to 7 C (19, 25). To search for the biochemical explanation of this temperature effect, leaves of cv. Salinas were kept at three different temperatures (0.5, 5.5, or 12.5 C) and the development of RS in relation to PAL activity was examined. At 5.5 C, PAL activity increased almost linearly, followed by the development of RS symptoms (Fig. 5a). At 12.5 C (Fig. 5b), PAL activity increased earlier than at 5.5 C, but subsequently leveled off and finally fell to a low level. Concomitant with the sharp reduction of PAL activity, the rate of increase of RS leveled off. One of the possible reasons for RS symptoms often becoming more severe at 5.5 C than at 12.5 C may be that a decay of PAL activity occurs at the higher temperature. This view is supported by the data shown in Figure 6. In the midcourse (day 4) of incubation at 5.5 C, half of

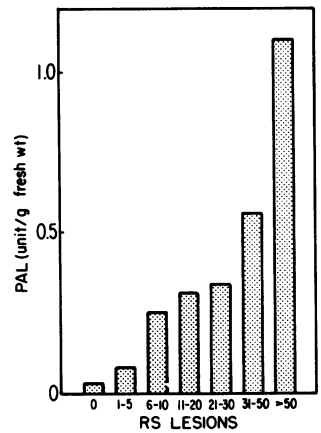

FIG. 4. Relationship between the level of PAL activity and the number of RS lesions. Five discs (1 cm in diameter), each of which had a number of RS lesions within the range specified, were taken randomly from three lettuce heads of an unknown cultivar which had been treated with ethylene for 7 days at 5.5 C.

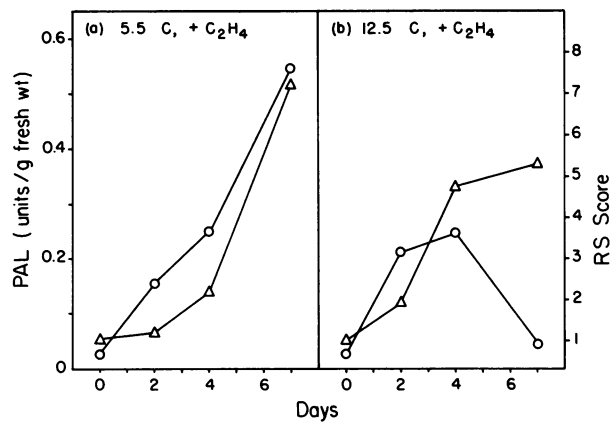

FIG. 5. Increase in PAL activity and development of RS in ethylene-treated leaves of cv. Salinas kept at 5.5 C (a), or 12.5 C (b). Δ: RS score; O: PAL activity.

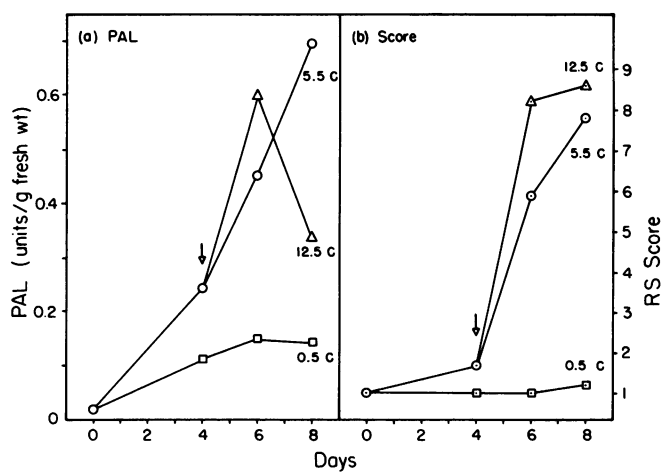

FIG. 6. Increase in PAL activity (a) and development of RS (b) in ethylene-treated leaves of cv. Salinas kept at three different temperatures. □: continuous 0.5 C; O: continuous 5.5 C; Δ: after incubation at 5.5 C for 4 days, the leaves were transferred to 12.5 C as indicated by arrow.

the leaves were transferred to 12.5 C. The rate of increase in PAL activity was first accelerated at the higher temperature but subsequently declined. Over the same period at 5.5 C, PAL activity was still increasing. At 0.5 C, PAL activity remained low throughout the incubation period and little RS occurred.

DISCUSSION

We have shown that when RS developed in response to ethylene treatment, phenolic compounds, particularly CHA and ICHA, increased significantly in the midribs of the affected leaves. The presence of CHA and ICHA in lettuce leaves has been reported (26). PPO and/or peroxidase may well participate in the development of RS by oxidizing the accumulated phenolics into quinones which subsequently form dark-colored polymers contributing to the pigmentation of RS. However, neither of them could have functioned as a rate-limiting enzyme in the development of RS because no correlation between RS formation and increase in activity of either PPO or peroxidase was observed. If metabolism of phenolics is involved in the development of RS, it is most likely that the supply of phenolics rather than the oxidation of phenolics is the rate-limiting factor.

Since the discovery of PAL by Koukol and Conn (12), many studies on physiological and biochemical aspects of this enzyme have been reported. The activity of PAL is often correlated with changes in the rate of accumulation of phenylpropanoids. This correlation suggests a possible causal relationship between the two biochemical events, and that PAL is the limiting factor in the biosynthetic pathways of such phenylpropanoids (2, 30). The induction of PAL is influenced by a variety of stimuli including light, plant hormones, wounding, and disease (2), and is often characterized by the concomitant development of a PAL inactivation system (2, 29). Ethylene has been known to induce PAL synthesis in a variety of plant tissues (3, 8, 11, 21, 23), although the response is not a general one. Many plant tissues do not develop PAL in response to ethylene, and in some plant tissues it was found that ethylene suppressed PAL development (5, 9, 27). In the present study, it was observed that different cultivars of lettuce showed very different responsiveness to ethylene in relation to the development of PAL, and this difference was closely correlated with their varietal susceptibility to RS formation. Although the genetic difference between cvs. Salinas and Calmar is not clear, the two varieties provide valuable materials for investigation of the physiology of RS.

Pronounced increases in PAL activity occurred following ethylene treatment, and these increases coincided with increases in RS. The increase in PAL activity preceded the formation of RS, suggesting that PAL may be involved in this process and is one of the rate-limiting factors for RS.

There is considerable variation in the extent of RS and PAL activity within the tissue under the same treatment. Where RS developed, higher PAL was also observed. However, it is not clear why some areas of the same leaf are more susceptible to PAL induction and RS development than others. These data suggest that not all of the cells in the midrib tissues, from the same head, or even the same leaf, are affected equally or concurrently by ethylene. There must be other factors which vary among cells and which participate in the regulation of PAL induction in response to ethylene. The molecular mechanism by which ethylene brings about an induction of PAL is unknown.

If the accumulation of phenolics, such as CHA or ICHA, is the cause of RS, it is expected that the administration of CHA or ICHA would yield RS without ethylene treatment. Such an experiment has been conducted, but with negative results. It may be that these substrates did not appreciably enter the appropriate sites within the cells. Alternatively, it is possible that the development of RS is independent of the accumulation of CHA or ICHA, but that both processes depend on the induction of PAL activity.

In this system we have demonstrated that CAH and OMT, as well as PAL, increased as a result of ethylene treatment. The concomitant increase of CAH and PAL in response to ethylene (7, 8) and other stimuli (1, 15) has been reported. Since the development of PAL is repressed by cinnamic acid and subsequent phenolic products (14, 30), the concomitant increases in the level of enzymes subsequent to PAL in the pathway of phenylpropanoid metabolism may play an important role in modulating the synthesis of PAL.

Anatomical study revealed that lignification occurred in RS-affected cells (R. Ilker, unpublished observation). Since the biosynthetic pathways of phenolics and lignins are interrelated, it is possible that lignification takes place in cells where phenolics accumulate.

It has been reported (19, 25), and the present study has confirmed that the optimal temperature for RS development in response to ethylene is near 5 C, and that at near 0 C RS does not occur. While PAL activity increased more rapidly at a higher temperature (12.5 C), it also decayed more rapidly. At 5.5 C, in contrast, no apparent decay of PAL was observed during the course of incubation. The decay of PAL at higher temperature (12.5 C) provides a reasonable explanation of why PAL activity, and consequently RS symptoms, were greater at 5.5 C than at 12.5 C. Since the development of a PAL inactivation system is known to occur following the PAL induction process in many plant tissues (4, 29), it is probable that decay in the present system may result from a similar relationship. Inhibition of the decay of induced PAL by lower temperature treatment has been noted in gherkin seedling tissues (4) and in tomato tissues (22). At 0.5 C little increase in PAL and no RS symptoms were observed. This may reflect failure of the tissue to respond to ethylene at low temperature.

Thus, an increase in PAL preceded and was parallel to RS formation; the extent of RS was directly related to the level of PAL; the RS-resistant variety had little capability to develop PAL in response to ethylene; and a temperature favorable for RS formation was also favorable for PAL development. These observations suggest that induction of PAL is closely interrelated with development of RS in response to ethylene, and PAL may serve as a useful biochemical marker for the RS reaction.

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